Transcriptional regulators CITED2 and PU.1 cooperate in maintaining hematopoietic stem cells

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Abstract

Reduced expression of the transcription factor PU.1 is frequently associated with development of acute myeloid leukemia (AML), whereas elevated levels of CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) enhance maintenance of both normal and leukemic hematopoietic stem and progenitor cells (HSPCs). Recent findings indicated that PU.1 and CITED2 act in the same gene regulatory network and we therefore examined a potential synergistic effect of simultaneous PU.1 downregulation and CITED2 upregulation on stem cell biology and AML pathogenesis. We found that simultaneous PU.1/CITED2 deregulation in human $CD34^+$ cord blood (CB) cells, as well as CITED2 upregulation in preleukemic murine PU.1-knockdown ($PU.1^{KD/KD}$) bone marrow cells, significantly increased the maintenance of HSPCs compared to the respective deregulation of either factor alone. Increased replating capacity of $PU.1^{KD/KD}$/CITED2 cells in *in vitro* assays eventually resulted in outgrowth of transformed cells, while upregulation of CITED2 in $PU.1^{KD/KD}$ cells enhanced their engraftment in *in vivo* transplantation studies without affecting leukemic transformation. Transcriptional analysis of $CD34^+$ CB cells with combined PU.1/CITED2 alterations revealed a set of differentially expressed genes that highly correlated with gene signatures found in various AML subtypes. These findings demonstrate that combined PU.1/CITED2 deregulation induces a transcriptional program that promotes HSPC maintenance which might be a pre-requisite for malignant transformation.

Highlights

- Simultaneous PU.1 down- and CITED2 upregulation increases human HSPCs maintenance
- CITED2-overexpression enhances maintenance of murine pre-leukemic $PU.1^{KD/KD}$ HSPCs
- Gene expression changes in PU.1-low/CITED2-high cells overlap with AML-signatures

Introduction

Leukemic transformation has been shown to be a multistep process in which hematopoietic cells acquire multiple mutations/alterations along the differentiation-road that can either influence self-renewing-, differentiation- or proliferation properties of cells.[1] Initial mutations are thought to occur in hematopoietic stem and progenitor cells (HSPCs) which can alter their lifespan and/or maintenance and lead to clonal hematopoiesis.[2,3] Additional mutations in such preleukemic HSPCs promote leukemic progression.[4] A key regulator of hematopoiesis is the ETS-family transcription factor PU.1 that is expressed at low levels in HSPCs[5] and at high levels in the myeloid linage and B-cells.[6–8] PU.1 has crucial functions for both HSPC-maintenance[9–14] and differentiation of the myeloid lineage.[15,16] In acute myeloid leukemia (AML), PU.1 expression is frequently found to be disturbed by mutations, translocations and changes in signal transduction[17–23], which contributes to the accumulation of immature blasts- the characteristic feature of AML. Notably, heterozygous mutations of the *SPI1* gene itself (which is the gene encoding PU.1) are only rarely found in human AML[24,25] and homozygous mutations are not detected at all, which is in line with murine models demonstrating that total absence of PU.1 is not compatible with hematopoiesis- whether it is healthy or pathologic.[11,26–29] To resemble the situation observed in patients, mouse models with reduced PU.1 expression rather than full *Spi1*-deletions have become useful models to study AML pathogenesis.[12,30–32] Homozygous deletions of a -14-kb upstream regulatory region (URE) in the Spi1 locus results in 80% reduction of PU.1 expression in murine bone marrow cells and mice develop AML at approximately 6 months of age. Malignant transformation in PU.1-knockdown mice was found to be recurrently accompanied by chromosomal aberrations[30], indicating that PU.1-low cells are more vulnerable for acquiring additional changes that promote leukemia development. Since PU.1 knockdown mice undergo a preleukemic phase of several months they can also serve as a model for studying alterations that precede leukemic transformation and identify cooperative

factors that accelerate or facilitate transformation. In particular, additional alterations that lead to an increased maintenance of PU.1-low HSPCs could contribute to expansion of a cell pool that is susceptible to mutation acquisition and thereby promote AML development.

We recently demonstrated that PU.1 negatively regulates the expression of the transcriptional co-activator CITED2 (CBP/p300-interacting-transactivator-with-an-ED-rich-tail 2) by binding to multiple ETS-binding sites in the CITED2 promoter.[33] CITED2 is a key guardian of hematopoietic stem cell (HSC) maintenance and its deletion in murine HSC results in increased cell apoptosis, cycling and consequently multi-lineage bone marrow failure.[34–36] Notably, CITED2 has also important functions for the survival of leukemic stem cells[33,37] and pathways that are involved in upregulating CITED2 expression[37–42] are frequently activated in AML. Therefore, we studied the combined de-regulation of PU.1 and CITED2 in normal and leukemic HSPCs.

Here we show that simultaneous upregulation of CITED2 and downregulation of PU.1 in human $CD34^+$ cord blood cells using lentiviral constructs enhances the maintenance of hematopoietic stem and progenitor cells (HSPC). Similar, CITED2 overexpression in preleukemic murine PU.1-knockdown bone marrow cells increased replating capacity and enhanced engrafted cells in transplantation assays, without affecting the transforming event. In summary, our data indicate that combining downregulation of PU.1 and upregulation of CITED2 enhances the lifespan of PU.1-low HSCs, which makes them more prone to full leukemic transformation.

Material and Methods

Isolation of stem- and progenitor cells

Neonatal cord blood was derived from healthy full-term pregnancies after informed consent from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands. Mononuclear cells were isolated by density gradient centrifugation using

Lymphoprep (Alere Technologies AS, Oslo, Norway) and $CD34^+$ cells were selected using the MACS CD34 microbead kit on autoMACS (Miltenyi Biotec, Leiden, The Netherlands). Lentiviral constructs and transduction procedure are described in the Supplementary information.

CFC assay

Transduced human $CD34^+$ cord blood cells were directly sorted in MethoCult H4230 (StemCell Technologies, Grenoble, France) supplemented with 19% (v/v) IMDM, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL SCF (Novoprotein) and 1 U/mL EPO (EPREX). Murine BM cells were isolated from 8-12 weeks old B6.J $URE^{-/-}$ mice.[30] Lineage depleted murine BM cells were transduced as described above and c-Kit^+ cells were directly sorted into MethoCult H4230 (StemCell Technologies) supplemented with 19% (v/v) IMDM (Lonza, Breda, The Netherlands), 100 ng/ml mSCF (PepProtech), 20 ng/ml hGM-CSF, 2 ng/ml mIL-3 (PepProtech). Colonies were scored after 12-14 days of incubation. Subsequently, 50000 cells were replated and again scored after 12-14 days.

Long-term cultures on stroma

Murine MS5 cells were expanded and cultured as described earlier.[43] Long-term Culture-Imitating Cell (LTC-IC) assays were performed by plating transduced $CD34^+$ cord blood cells in limiting dilutions in the range of 9 to 1000 cells per well on MS5 stromal cells in 96-well plates in LTC medium (αMEM supplemented with heat-inactivated 12.5% FCS, heat-inactivated 12.5% horse serum (Sigma, Zwijndrecht, The Netherlands), 100 U/mL penicillin/streptomycin, 200 mM glutamine, 57.2 μM β-mercaptoethanol [Sigma] and 1 μM hydrocortisone [Sigma]). After 5 weeks, methylcellulose (MethoCult H4230 supplemented with 19% (v/v) IMDM, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL SCF and 1 U/mL EPO) was added to the wells. Two weeks later, wells containing CFCs were scored as positive. LTC-iC frequency was calculated using the L-Calc software. For MS5 co-culture growth curves, 10-50 $x10^5$ cells transduced

$CD34^+$ cord blood cells were plated on MS5 stromal cells in a T25 culture flask in LTC medium supplemented with 20 ng/ml IL-3, G-CSF and TPO. Cultures were demi-depopulated weekly for analysis.

In vivo transplantations into NSG mice

Murine $Lin^-c\text{-}Kit^+$ BM cells were isolated from 8-12 weeks old B6.J $URE^{-/-}$ mice by means of lineage depletion (Dynabeads), followed by c-Kit enrichment (MACS). Cells were resuspended in M5300 medium (StemCell Technologies) supplemented with rmSCF (50 ng/ml), rmTPO (20 ng/ml), rmIL3 (25 ng/ml), rmIL6 (10 ng/ml) and Primocine (anti mycoplasma agent 2ul/ml). The next day, the cells were in 2 subsequent rounds lentivirally transduced with GFP-tagged control or CITED2 overexpressing lentivirus in the presence of 4 ng/ml polybrene. After 2 days, 0.2×10^6 cells for cohort A and 0.5×10^6 cells for cohort B were retro-orbitally injected into NSG mice. Before transplantations, mice were sublethally irradiated (2.0 Gy). Engraftment was analyzed in the peripheral blood (PB) and bone marrow (BM) by flow cytometry.

Gene expression profiling

From 4 independent cord blood batches, $CD34^+$ cells were MACS isolated and transduced with control lentivirus, CITED2 overexpressing lentivirus, a shRNA lentivirus against PU.1 or a lentivirus containing a CITED2 overexpression cassette and a shRNA against PU.1. After 2 days transduced $CD34^+$ were sorted from each transduction group (Group 1: Control; Group 2: CITED2; Group 3: shPU.1; Group 4: CITED2/shPU.1). Total RNA was isolated using the RNeasy mini kit from Qiagen (Venlo, The Netherlands) according to the manufacturer's recommendations. Q-PCR analysis was used to validate proper overexpression or knock-down of CITED2 and PU.1 respectively. RNA from 2 cord bloods with similar overexpression or knock-down of CITED2 and PU.1 was pooled within each group and quality was examined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Genome-wide

expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA, USA) BeadChip Arrays (Illumina HT12-V4). Typically, 0.5–1 µg of mRNA was used in labeling reactions and hybridization with the arrays was performed according to the manufacturer's instructions. The expression was quantile normalized using GeneSpring GX software, and from the probesets that were expressed above background (set to 25) subsequent fold differences were calculated. Genes that indicated a fold-change of <2 or >2 were further analyzed. Array data are available at http://www.ncbi.nlm.nih.gov/geo, with accession code: GSE118036. Array data were compared to publically available (http://servers.binf.ku.dk/bloodspot/) gene expression data from 198 AML patients (60 t(8;21); 47 inv(16)/t16;16); 43 t(11q23) and 48 AMLs with complex karyotype) and 18 normal $CD34^+$ subsets.

Immunoblotting

Preparation of cell lysates and immunoblotting procedure was performed as described previously.[44] Primary antibodies for immunoblotting were: MRG1 (JA22, Santa Cruz, #SC-21795), PU.1 (T-21, Santa Cruz, #SC-352).

Statistical analysis

If not indicated otherwise in figure legends, p-values were calculated using the students t-test.

Results

Combining PU.1 down-regulation with CITED2-upregulation maintains HSCs

In order to investigate the impact of combined deregulation of PU.1- and CITED2 levels on HSPCs, $CD34^+$ cord blood (CB) cells were isolated and double-transduced with various combinations of lentiviral constructs to achieve either short hairpin (sh)-mediated PU.1

downregulation, CITED2 upregulation or a combination of both. For all conditions, we observed 20-25 percent double-transduced cells, and three days after transduction, cells were sorted and plated for colony forming cell (CFC) assays (Figure 1A, Supplementary Figure S1A-B). Levels of PU.1 reduction and CITED2 overexpression were confirmed by both Q-PCR and western blot (Figure 1B, Supplementary Figure S1C). The shPU.1-, CITED2- and shPU1/CITED2-transduced cells provided comparable CFU-GM, BFU-E and CFU-GEMM colony formation compared to control cells (Figure 1C-D). However, replating experiments showed increased replating capacity of shPU.1/CITED2 cells which could not be achieved by altering levels of PU.1 or CITED2 only (Figure 1E). Furthermore, we showed that the enhanced replating capacity of shPU.1/CITED2 cells is restricted to the $CD34^{+}CD38^{-}$ fraction (Figure 1F-G), which suggests that it is primarily the more immature fraction of HSPCs that is maintained by simultaneous alterations of CITED2- and PU.1 expression levels.

Simultaneous PU.1 down and CITED2 upregulation increases LTC-iC frequency

To address the question if combined PU.1/CITED2 deregulation can also impact long-term functions of HSPCs, control-, shPU.1-, CITED2- and shPU1/CITED2- transduced $CD34^{+}$ CB cells were cultured on a MS5 stromal layer in the presence of cytokines for up to 4 weeks and subsequently plated for CFC assays (Figure 2A). Total cell numbers of shPU.1-, CITED2- and shPU1/CITED2- cells in MS5 co-cultures were not different compared to control cells (Figure 2B), indicating that growth factor-induced HSPC expansion was not significantly affected by PU.1/CITED2 deregulation. Cells that were plated in methyl cellulose after 3 or 4 weeks of culturing formed equal number of colonies in CFC assays (Figure 2C), however, replating of CFC assays with shPU1/CITED2 cells resulted in a significantly higher number of colonies compared to all other conditions (Figure 2D). Remarkably, colony formation in 2^{nd} round of replating was solely restricted to shPU1/CITED2 cells (Figure 2D). The impact of PU.1/CITED2

deregulation on HSPC maintenance was further evaluated by performing Long-Term Culture-initiating Cell (LTC-iC) assays, in which cells are maintained for 5 weeks on a MS5 stromal layer without additional growth factors prior to reading out their colony forming capacity (Figure 2E). Whereas PU.1 downregulation did not alter the LTC-IC frequency significantly compared to control cells, upregulation of CITED2 resulted in a 4-fold increase, and combined shPU.1/CITED2 alteration in an 8-fold increase of the LTC-iC frequency (Figure 2F-G). These data indicate that upregulation of CITED2 alone can be sufficient to increase HSPC maintenance under certain conditions. However, if HSPC maintenance is challenged by external signals such as activation of signalling cascades that promote cell proliferation or differentiation, simultaneous downregulation of PU.1 and upregulation of CITED2 can increases the HSPC frequency more effectively.

CITED2 overexpression in PU.1$^{KD/KD}$ bone marrow cells enhances the outgrowth of immature cells in *in vitro* replating assays

To confirm and study the effects of CITED2 upregulation in cells with low PU.1 expression with an alternative strategy, we lentivirally overexpressed CITED2 in murine PU.1$^{KD/KD}$ bone marrow (BM) cells, which have the potential to transform and are therefore pre-leukemic.[30] Reduction of PU.1 expression in PU.1$^{KD/KD}$ cells compared to PU.1$^{WT/WT}$ cells was confirmed by Q-PCR (Supplementary Figure S2A). Lineage-depleted PU.1$^{KD/KD}$ BM cells were isolated from mice in a pre-leukemic phase (n=6) and control- or CITED2 transduced cells were sorted in methylcellulose to perform CFC-assays with subsequent replating (Figure 3A). Similar to the experiments performed with CB cells, we observed comparable numbers of CFC's in control- and CITED2 transduced cells in the primary CFC assay, whereas CITED2 overexpression resulted in significantly more colonies following the 1st replate ($p<0.05$; Figure 3B). Phenotypically we did not observe differences in control- vs. CITED2 colonies (Supplementary

Figure S2B). Interestingly, in the 2nd and 3rd replate, samples could be divided in 2 groups based on colony number and replating ability. With cells obtained from 4 mice (group 1), colony formation could be observed in 3 rounds of replates with higher colony numbers in CITED2- compared to control samples (in average 69 vs 5 colonies in 2nd replate, p=0.05; Figure 3B). In group 2 both control- and CITED2-transduced cells gave rise to several hundred colonies even in a 3rd replate (Figure 3B). Notably, group 1 and 2 showed similar transduction efficiencies at the moment of cell sorting. Cytospins of CFC's showed presence of mature cells in the 1st replate of group 1, whereas the 3rd replate was dominated by cells with immature morphology with a number of blast cells (Figure 3C). In contrast, a phenotypically homogenous population of immature blast cells was already present in the 1st replates of group 2 (Figure 3C), which contained c-Kit^{pos}/Gr-1^{neg} and c-Kit^{pos}/Gr-1^{low} cell populations (Supplementary Figure S2C). By flow cytometric analysis for c-Kit and Sca-1 expression, we observed that colonies from replates of control cells mainly consist of a rather homogenous c-Kit^{low}Sca-1^{neg} cell population (Figure 3D), whereas colonies from CITED2-transduced cells showed a more heterogeneous pictures consisting of c-Kit^{low}, c-Kit^{high}, and c-Kit^{high}/Sca-1^{pos} cells (Figure 3D-E). Based on these data we concluded that CITED2 is not required or additive to leukemic transformation, but potentially supports the outgrowth of phenotypically immature PU.1$^{KD/KD}$ cells, at least for the time frame we performed the experiments.

Overexpression of CITED2 in PU.1$^{KD/KD}$ is not sufficient for leukemia initiation *in vivo*

Next, we questioned whether overexpression of CITED2 in murine PU.1$^{KD/KD}$ cells contributes to leukemia development *in vivo*. Therefore, c-Kit^{pos}-HSPCs were isolated from PU.1$^{KD/KD}$ donor mice and transduced with control or CITED2 overexpressing lentivirus (Supplementary Figure S3A). Subsequently, transduced cells were transplanted into irradiated NSG recipient mice (Figure 4A). Two independent experiments (referred to as cohort A and B) were performed with

each cohort consisting of 7 mice receiving control-transduced cells and 7 mice receiving CITED2-transduced cells. In both cohorts, we observed a significantly higher percentage of CITED2-GFP donor cells compared to control-GFP donor cells in the peripheral blood of recipient mice 11-15 weeks after injection (Figure 4B). However, only modestly higher levels of CITED2-GFP compared to control-GFP donor cells were found in the bone marrow of recipient mice after 34 weeks (cohort A) and 24 weeks (cohort B) respectively (Figure 4C), which were non-significant differences. In none of the mice signs of leukemia development were observed, as indicated by normal weight of spleen and liver (Figure 4D, Supplementary Figure S3D). These data indicate that CITED2-GFP PU.1$^{KD/KD}$ donor c-Kit^{pos}-HSPCs contribute faster to engraftment than control-GFP PU.1$^{KD/KD}$ cells, however, CITED2 overexpression does not enhance the initiation of leukemia within this time frame.

shPU.1/CITED2-induced gene expression patterns correlate with gene expression profiles observed in AML

In order to investigate the transcriptional changes caused by shPU.1/CITED2 gene deregulation, an Illumina BeadChip array was performed with CD34^{+} CB cells transduced with the corresponding lentiviral vectors (Figure 5A, Supplementary Figure S4A-B). PU.1 downregulation and CITED2 overexpression of sorted cells was verified by Q-PCR (Supplementary Figure S4C). We found that downregulation of PU.1, overexpression of CITED2 or the combination of both respectively led to 176, 205 or 148 differentially expressed genes (>2-fold up- or downregulated in both replicates, Figure 5B), as compared to control transduced cells. Notably, the 148 probe sets that were found differently expressed in shPU.1/CITED2 cells, were partly overlapping with deregulated genes found in shPU.1- or CITED2 only cells (32/148 overlap with shPU.1; 35/148 overlap with CITED2), but also contained a unique set of genes (94/148). (Figure 5B, Supplementary Table S1). In general, gene expression changes were surprisingly modest and

unexpectedly, both pathway- and GSEA analysis did not reveal significant signatures linked to stem cell maintenance or cell proliferation. Despite these findings, we decided to explore whether gene expression changes induced by combined shPU.1/CITED2 deregulation overlap with changes observed in $CD34^+$ AML cells in comparison to normal $CD34^+$ cells. We therefore downloaded gene expression data from 198 AML patients and 18 normal $CD34^+$ subsets from the BloodSpot database (http://servers.binf.ku.dk/bloodspot/). 112/148 probesets from our study could be linked to a gene, of which 67 were present in the BloodSpot database. Of these 67 genes, 34 were upregulated and 33 genes were downregulated in shPU.1 /CITED2-transduced cells from our study. Notably, we observed that the majority of genes that were found upregulated in our shPU.1/CITED2 cells are also upregulated in AML patients (29/34), whereas 14 out of 33 downregulated genes in shPU.1/CITED2 cells are also found downregulated in AML patients when compared to normal $CD34^+$ cells (Figure 5C). A Spearman's ranked correlation analysis demonstrated that the gene expression changes we observed in shPU.1/CITED2 cells significantly correlated with the changes observed in AML (Figure 5D), with the highest correlation observed for upregulated genes. The overlap of gene expression patterns of shPU.1/CITED2 cells and AML patients was not specific for a certain subtype of AML but could be found across various AML patients, suggesting that the modest transcriptional changes caused by combined PU.1/CITED2 deregulation could be generally supportive for AML development when combined with variable additional hits.

Discussion

In the present study we demonstrated that combined upregulation of CITED2 and downregulation of PU.1 increases HSPC maintenance using two alternative approaches. Simultaneous overexpression of CITED2 and knockdown of PU.1 in $CD34^+$ cord blood cells

using lentiviral vectors resulted in enhanced replating capacity in CFC-assays and increased the LTC-IC frequency. Similar results were obtained when CITED2 was upregulated in preleukemic murine PU.1$^{KD/KD}$ c-Kit^{pos}-HSPCs.

AML is characterized by a stepwise accumulation of genetic and epigenetic alterations that first result in the generation of a clonal and/or preleukemic state before eventually leading to fully transformed leukemic cells. Altered regulation of self-renewal, maintenance and proliferation without a block in differentiation have been described as an early event in malignant transformation.[2,3,45–47] Recently, several studies have shown that clonal hematopoiesis with driver mutations can be detected in a large cohort of elderly patients whereby clonal cells outcompete the remaining cells. However, only a limited number of these patients develop AML, in particular when co-mutations occur, thereby triggering alternative pathways and making the cells prone for AML transformation.[4,3,48] Apparently, cord blood shPU.1/CITED2 HSCs mimic the initial step in clonal evolution, reflected by increased replating capacity, increased LTC-IC frequency, but not a block in differentiation.

Since CITED2 expression is found upregulated in AML and was shown to be essential for leukemic cell survival[33,37] we wondered if CITED2 overexpression in definite preleukemic cells contributes to their transformation. Knockdown of PU.1 in murine HSCs results in AML development after undergoing a preleukemic phase of several months[30,31], and therefore untransformed PU.1$^{KD/KD}$ cells resemble such a condition. The results of the present study demonstrate that overexpression of CITED2 in untransformed PU.1$^{KD/KD}$ c-Kit^{pos}-HSPCs is not sufficient for immediate leukemia onset, however, expands the pool of preleukemic PU.1$^{KD/KD}$ HSPCs. An interesting question that could be addressed in future studies is whether upregulation of CITED2 in PU.1$^{KD/KD}$ cells prior to (serial) transplantation-experiments would have an impact on AML development or LSC maintenance when combined with additional alterations. Other mutations have been identified that accelerate the process of leukemic

316 transformation when combined with PU.1 downregulation. For instance, mice carrying a
317 mutation in K-Ras rapidly progress from a myeloproliferative neoplasm to an aggressive AML
318 when deleting a deubiquitylase that regulates PU.1 stability.[23] In addition, in mice with a
319 homozygous deletion of *Msh2*, a gene involved in DNA mismatch repair, slight reductions in
320 PU.1 levels were shown to promote AML progression.[32] Similarly, reduction of PU.1 levels in
321 p53-/- mice resulted in AML development, which is not observed when only p53 is deleted.[49]
322 Mechanistically, the importance of CITED2 in maintaining both HSCs and LSCs has been linked
323 to cell apoptosis in a p53-dependent manner.[35] We have shown previously that loss of CITED2
324 triggers leukemic cells death trough stabilisation of p53,[44] an observation also made for other
325 types of cancer.[50] It is likely that the reverse might occur in the context of CITED2
326 overexpression, making HSC less sensitive to stress response pathways and facilitating the
327 process of stem cell maintenance.

328 The PU.1$^{KD/KD}$ HSPCs showed up to 80% reduction of PU.1 levels, whereas our lentiviral-
329 mediated PU.1 knockdown in cord blood cells ranged between 20%-50% reduction in PU.1
330 levels (Supp. Figure S4C). Strikingly, despite the variability of PU.1 downregulation, both genetic
331 models resulted in similar phenotypes. These data indicate that already a modest reduction of
332 PU.1 levels, which is also observed in AML cells,[18,19,21,22,24] can lead to an increased HSPC
333 maintenance in combination with elevated CITED2 levels. We therefore also aimed to get more
334 insight in the transcriptional changes observes in shPU.1/CITED2 cells. Gene expression
335 analysis revealed that there are a number of genes differentially expressed in shPU.1/CITED2
336 CD34$^+$ cord blood cells, which are not deregulated when only PU.1- or CITED2 levels are
337 altered, indicating that a unique transcriptional program is altered by combined shPU.1/CITED2
338 alteration. Furthermore, we found that gene expression changes in shPU.1/CITED2 CD34$^+$ cells
339 mimic a pattern found in patients with various AML subtypes, indicating that our genetic model of

combined PU.1/CITED2 deregulation resembles a state that is in general supportive for AML development.

In summary, our genetic models with combined CITED2/PU.1 deregulation mimic the initial step in clonal leukemia evolution and can serve as useful tools to further study and understand the molecular mechanism of AML development.

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Authorship and Conflict of Interest

Contribution: K.M. performed experiments, analyzed data, made the figures and wrote the manuscript. M.G. and P.M.K. performed experiments and analyzed data. A.Z.B. and R.S.N.F. helped with analyzing microarray data. T.I.T. isolated and provided primary murine PU.1$^{KD/KD}$ cells. U.S. provided PU.1$^{KD/KD}$ mouse strains and helped revising the manuscript. E.V. discussed results, provided critical advice and edited the manuscript. H.S. designed the study, performed experiments, interpreted results and revised the manuscript.

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503 cancer cells to cisplatin through stabilization of p53 and enhancement of p53-dependent
504 apoptosis. *J Cell Physiol*. 2011;226(9):2415-2428. doi:10.1002/jcp.22589.

505

506

Figure legends

Graphical abstract Simultaneous downregulation of the transcription factor PU.1 and upregulation of CITED2 increases the maintenance of hematopoietic stem and progenitor cells (HSPCs). Since cells with low PU.1 levels are considered to be potential preleukemic, combined PU.1/CITED2 deregulation increases the pool of HSPCs that might be prone for leukemic transformation.

Figure1 C**ombining PU.1 down-regulation with CITED2-upregulation maintains HSCs (A)** Schematic overview of experimental design. Isolated $CD34^+$ cord blood (CB) cells were transduced with indicated combinations of lentiviral constructs and double-positive cells were sorted for CFC assays **(B)** Downregulation of PU.1 and upregulation of CITED2 by our lentiviral vectors was verified by western blot in the Molm13 leukemic cell line. **(C, D)** CFC assays performed with transduced $CD34^+$ CB cells. Relative percentage of indicated colony types (C) and total number of colonies (D) scored after 14 days is shown. **(E)** Colonies from primary CFC assays were harvested and 50000 cells were replated. Total amount of colonies after 14 days is shown. **(F, G)** Colony number in CFC assays (F) and replates (G) of transduced $CD34^+CD38^-$ and $CD34^+CD38^+$ cell population. **(D-G)** Error bars indicate s.d. of 3 individual experiments performed in duplicates. Each experiment was performed with $CD34^+$ cells from 2-3 donors. n.s.= not significant; **P<0.01 compared to control;

Figure2 **shPU.1/CITED2 cells contain the highest LTC-IC frequency (A)** Schematic overview of experimental design. $CD34^+$ cord blood (CB) cells were transduced with indicated lentiviral constructs and plated on a MS5 stromal layer. After 3-4 weeks, CFC assays were performed from cultured cells. **(B)** Growth curve of transduced $CD34^+$ cells cultured on a MS5 stromal layer in Gartners medium. Error bars indicate s.d. of 6 individual experiments. Each experiment was

performed with CB from several donors. **(C)** CFC assays of transduced CB cells that have been cultured as in (B) for 3 or 4 weeks. Error bars indicate s.d. of 3 individual experiments performed in duplicates. n.s.= not significant. **(D)** 1st and 2nd replate of cells harvested from CFC assays shown in (C). Error bars indicate s.d. of 3 individual experiments performed in duplicates. n.s.= not significant, *P<0.05 **(E)** Experimental design of Long-term Culture-Initiating Cell (LTC-iC) assay: Transduced $CD34^+$ CB cells were sorted in a MS5-coated 96 well plate in limiting dilutions of 9-1000 cells. After 5 weeks, methylcellulose was added and wells were scored as positive or negative for colony forming units (CFU) after 14 days to determine LTC-iC frequencies. **(F)** Scoring of 1 representative LTC-iC experiment performed as described in (E). **(G)** Average LTC-IC frequency of 3 individual experiments is shown. Each individual experiment was performed with $CD34^+$ cells from serval donors. Error bars indicate s.d.; n.s.= not significant, *P<0.05.

Figure 3 **Overexpression of CITED2 in murine $PU.1^{KD/KD}$ bone marrow cells maintains stem- and progenitor cells prior to transformation (A)** Schematic overview of experimental design. Lineage depleted bone marrow (BM) cells derived from $PU.1^{KD/KD}$ mice were transduced with control or CITED2 overexpressing lentivirus and sorted cells were applied to CFC assays and subsequent replating. **(B)** Number of colonies in CFC assays and replates performed with $PU.1^{KD/KD}$ cells transduced with control- or CITED2 constructs. Data points connected by a black line belong to cells isolated from the same mouse. Data from 6 individual experiments using BM from 6 individual mice are shown. Samples were separated in 2 groups based on colony number, group 2 is labelled by a red border. *P<0.05. **(C)** May Grunwald/Giemsa staining of cells harvested from CFC assays performed in (B); scale bars: 10 µm. **(D)** Representative FACS plots indicating c-Kit and Sca-1 expression of lineage negative control- and CITED2 transduced $PU.1^{KD/KD}$ cells harvested from CFC assay replates shown in (B). Plots of 2 mice from group 2

are shown (#1, #2). Numbers in gates indicate percentage of c-Kit^{-}, c-Kit^{+}, c-Kit^{++} and c-Kit^{++}/Sca-1^{+} double pos. cell populations. **(E)** Graph indicating the percentage of c-Kit^{++} and c-Kit^{++}/Sca-1^{+} cell fractions observed by FACS analysis as described in (D); group 2 samples are labelled by a red border; n=5, *P<0.05.

Figure 4 **Overexpression of CITED2 in PU.1$^{KD/KD}$ HSCs is not sufficient for leukemic transformation (A)** Schematic overview of experimental design. Lin^{-}cKit^{+} BM donor cells from PU.1$^{KD/KD}$ mice were transduced with a GFP-tagged control or CITED2 lentivirus and retro-orbital injected into irradiated NSG recipient mice. 2 independent experiments (referred as cohort A and B) were performed with 7 control- and 7 CITED2- recipient mice each being injected. **(B)** Percentage of GFP^{+}CD45.2 cells at time of injection and in peripheral blood after indicated number of weeks is shown. Error bars indicate s.d.; n=7 for each cohort; *P<0.05. **(C)** Percentage of GFP^{+} cells in bone marrow of recipient mice at day of sacrifice 30-34 weeks after injection is shown. Error bars indicate s.d.; n=14; **(D)** Spleen weights of sacrificed mice is indicated; Error bars indicate s.d.

Figure 5 **Combining PU.1 down-regulation with CITED2-upregulation induces a gene expression pattern also observed in AML (A)** Schematic overview of experimental design. Isolated CD34^{+} cord blood cells were transduced with indicated lentiviral constructs and transduced CD34^{+} cells were sorted for Illumina BeadChip Arrays **(B)** VENN diagram indicating the number of genes changed >2-fold in duplicate arrays, compared to control transduced cells **(C)** Gene expression comparison of non-APL AMLs vs. normal CD34^{+} cells. Each column is an AML sample with the red squares at the top indicating the subtype. **(D)** Spearman's rank

579 correlation between genes that are differentially expressed in shPU.1/CITED2 cells and genes
580 differentially expressed in non-APL AMLs. ***P<0.005, ****P<0.001.

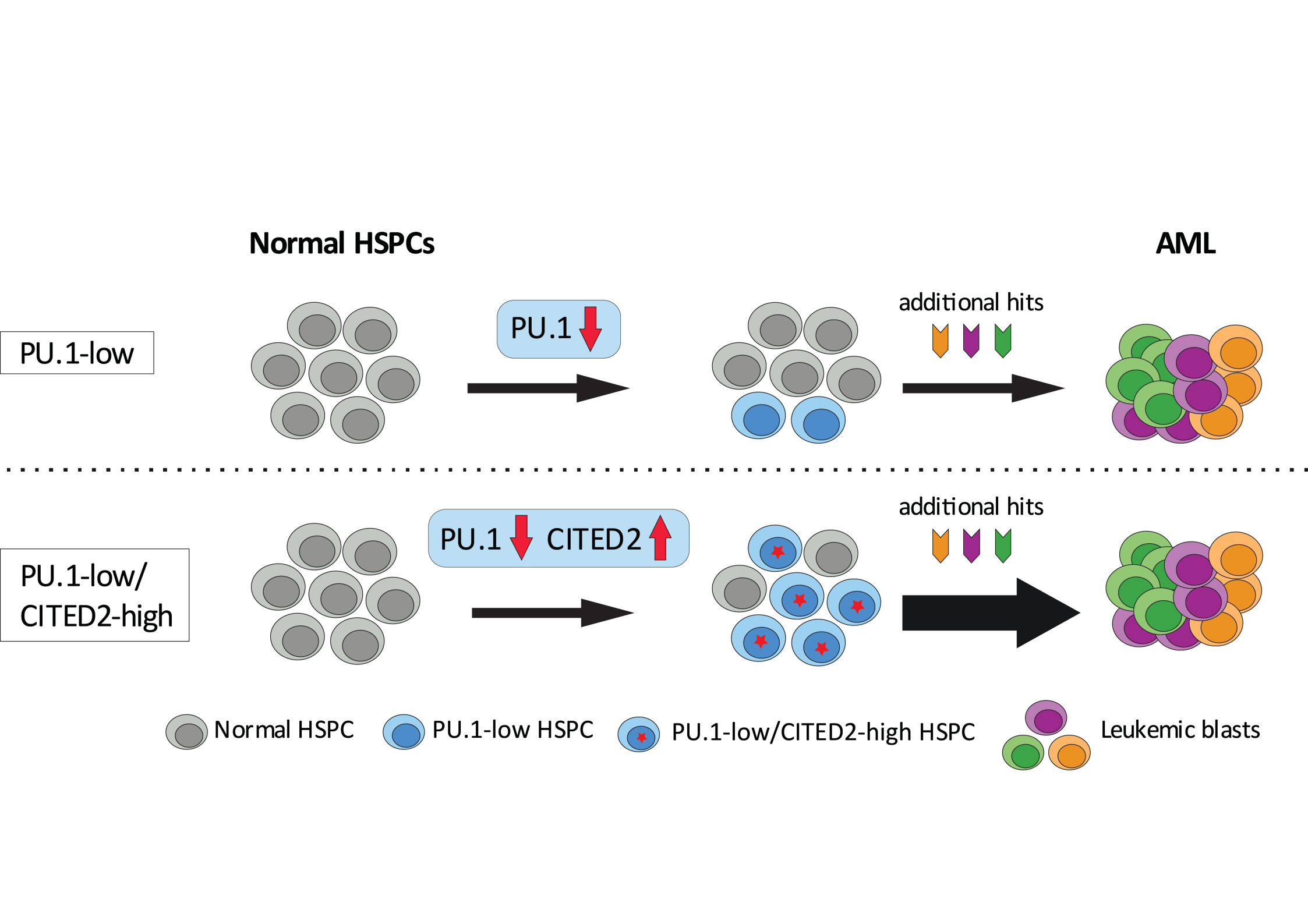

Normal HSPCs
AML
PU.1-low
PU.1
additional hits
PU.1-low/
CITED2-high
PU.1
CITED2
additional hits
Normal HSPC
PU.1-low HSPC
PU.1-low/CITED2-high HSPC
Leukemic blasts

Figure 1

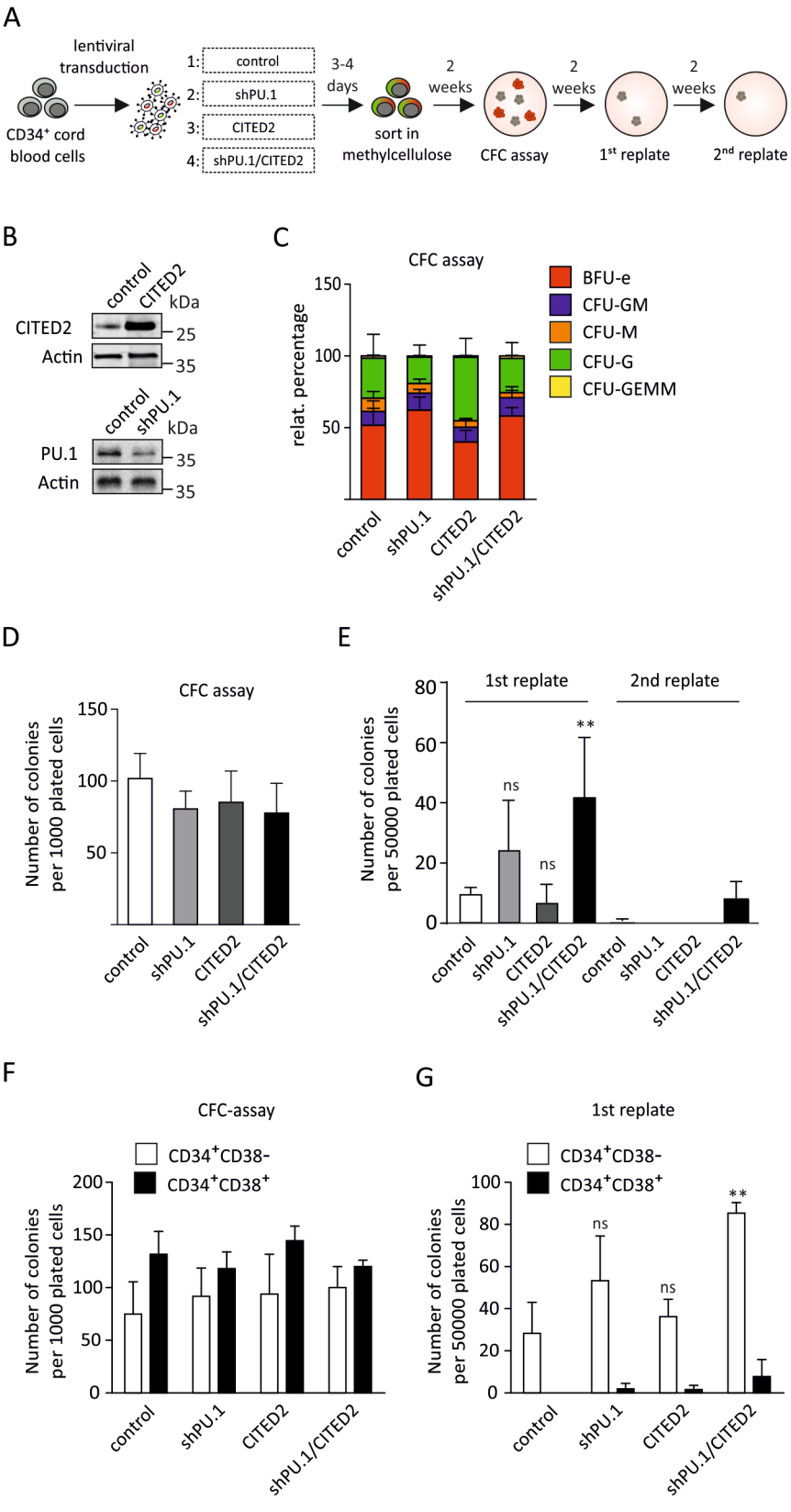

A
lentiviral transduction
CD34+ cord blood cells
1: control
2: shPU.1
3: CITED2
4: shPU.1/CITED2
3-4 days
sort in methylcellulose
2 weeks
CFC assay
2 weeks
1st replate
2 weeks
2nd replate
B
control
CITED2
kDa
CITED2
Actin
25
35
control
shPU.1
kDa
PU.1
Actin
35
35
C
CFC assay
relat. percentage
150
100
50
BFU-e
CFU-GM
CFU-M
CFU-G
CFU-GEMM
control
shPU.1
CITED2
shPU.1/CITED2
D
CFC assay
Number of colonies per 1000 plated cells
150
100
50
control
shPU.1
CITED2
shPU.1/CITED2
E
1st replate
2nd replate
Number of colonies per 50000 plated cells
80
60
40
20
0
ns
ns
**
control
shPU.1
CITED2
shPU.1/CITED2
control
shPU.1
CITED2
shPU.1/CITED2
F
CFC-assay
CD34+CD38-
CD34+CD38+
Number of colonies per 1000 plated cells
200
150
100
50
0
control
shPU.1
CITED2
shPU.1/CITED2
G
1st replate
CD34+CD38-
CD34+CD38+
Number of colonies per 50000 plated cells
100
80
60
40
20
0
ns
ns
**
control
shPU.1
CITED2
shPU.1/CITED2

Figure 2

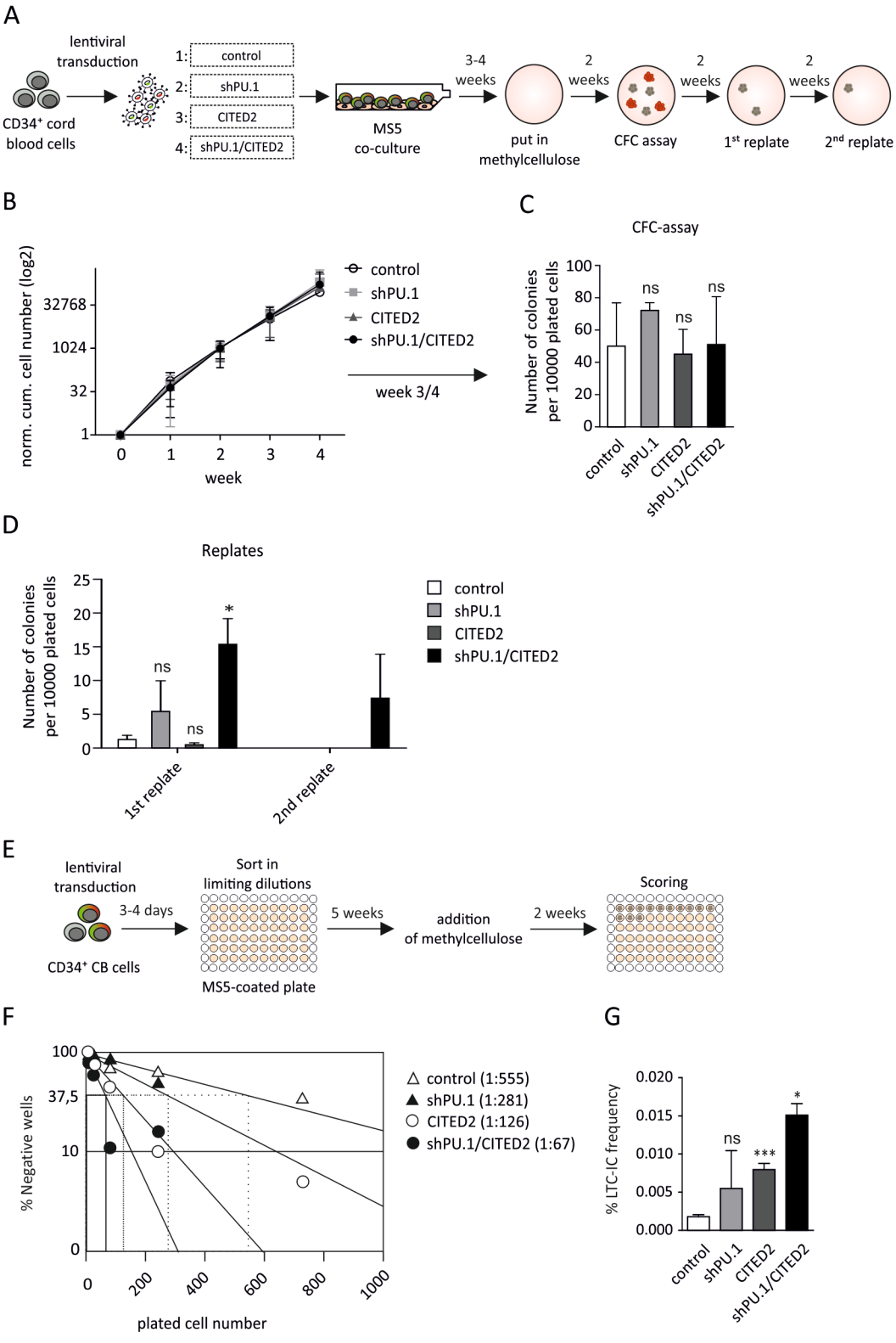
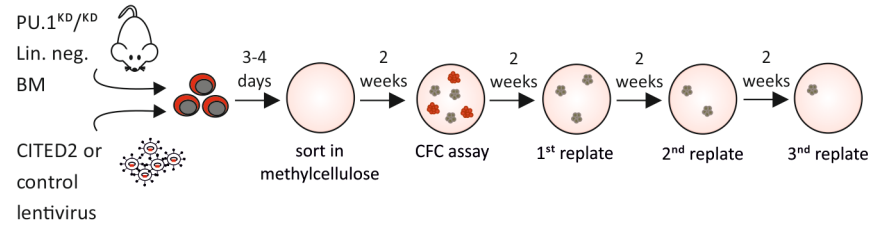
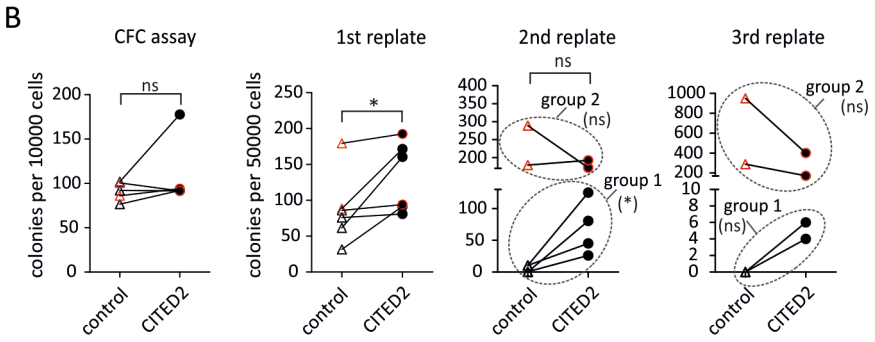
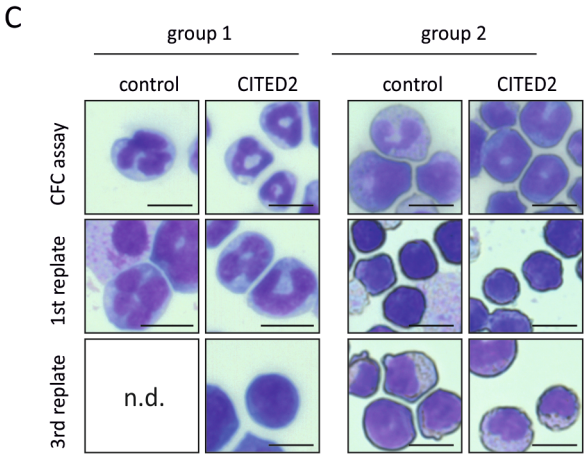
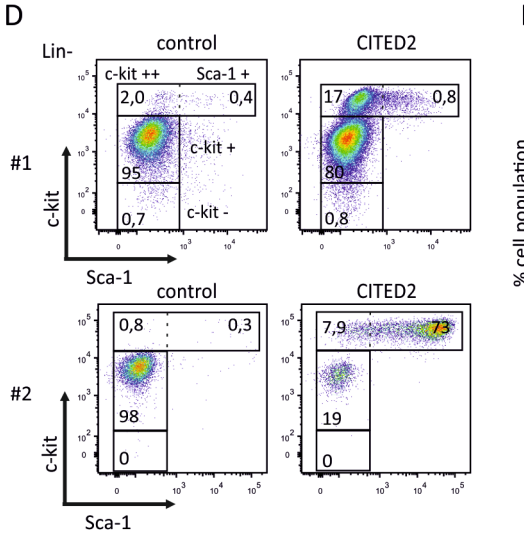

A
lentiviral transduction
CD34⁺ cord blood cells
1: control
2: shPU.1
3: CITED2
4: shPU.1/CITED2
MS5 co-culture
3-4 weeks
put in methylcellulose
2 weeks
CFC assay
2 weeks
1st replate
2 weeks
2nd replate
B
norm. cum. cell number (log2)
32768
1024
32
1
0 1 2 3 4
week
control
shPU.1
CITED2
shPU.1/CITED2
week 3/4
C
CFC-assay
Number of colonies per 10000 plated cells
100
80
60
40
20
0
ns
ns
ns
control
shPU.1
CITED2
shPU.1/CITED2
D
Replates
Number of colonies per 10000 plated cells
25
20
15
10
5
0
ns
*
ns
1st replate
2nd replate
control
shPU.1
CITED2
shPU.1/CITED2
E
lentiviral transduction
3-4 days
CD34⁺ CB cells
Sort in limiting dilutions
MS5-coated plate
5 weeks
addition of methylcellulose
2 weeks
Scoring
F
% Negative wells
100
37,5
10
0
0 200 400 600 800 1000
plated cell number
control (1:555)
shPU.1 (1:281)
CITED2 (1:126)
shPU.1/CITED2 (1:67)
G
% LTC-IC frequency
0.020
0.015
0.010
0.005
0.000
ns
***
*
control
shPU.1
CITED2
shPU.1/CITED2

Figure 3


A
PU.1KD/KD
Lin. neg.
BM
CITED2 or
control
lentivirus
3-4
days
sort in
methylcellulose
2
weeks
CFC assay
1st replate
2nd replate
3rd replate


B
CFC assay
1st replate
2nd replate
3rd replate
colonies per 10000 cells
colonies per 50000 cells
ns
*
group 1
group 2
(ns)
(*)
control
CITED2


C
group 1
group 2
control
CITED2
CFC assay
1st replate
3rd replate
n.d.


D
Lin-
control
CITED2
c-kit ++
Sca-1 +
c-kit +
c-kit -
2,0
0,4
95
0,7
17
0,8
80
0,8
#1
#2
c-kit
Sca-1
0,8
0,3
98
0
7,9
73
19
0

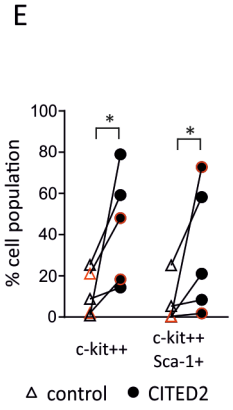

E
% cell population
*
c-kit++
c-kit++
Sca-1+
control
CITED2

Figure 4

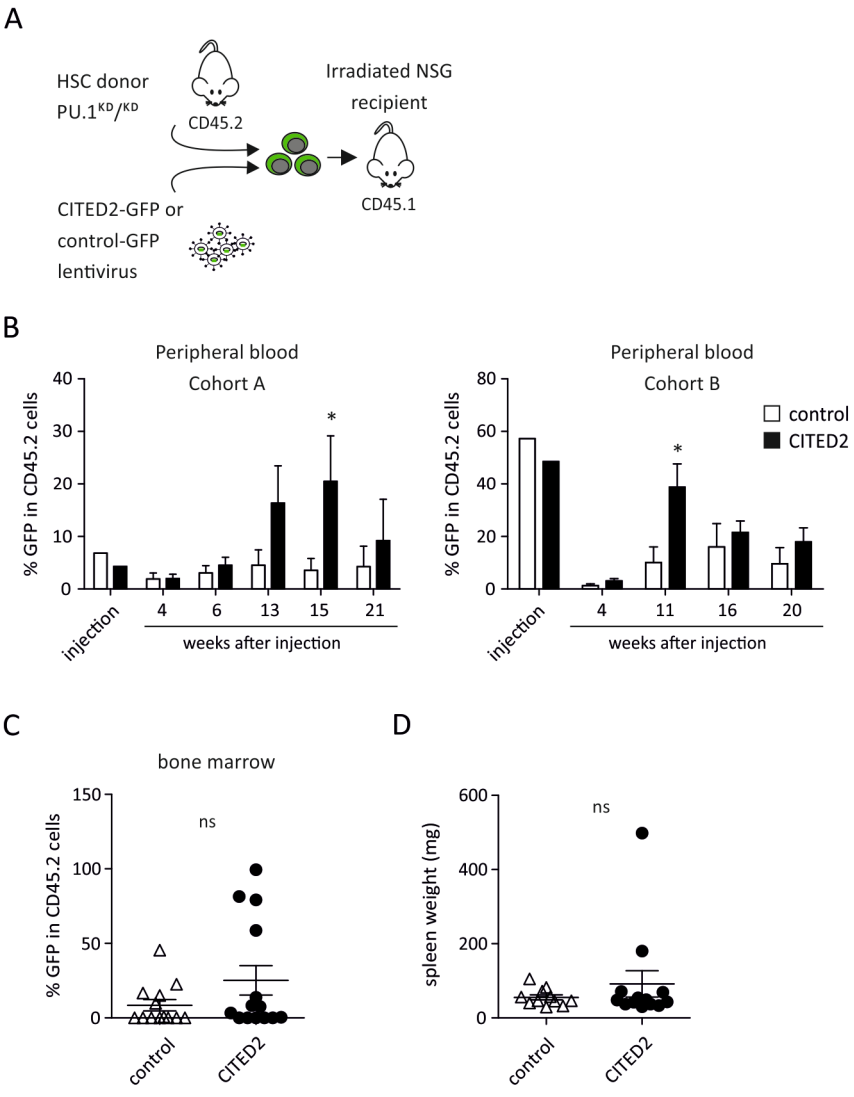
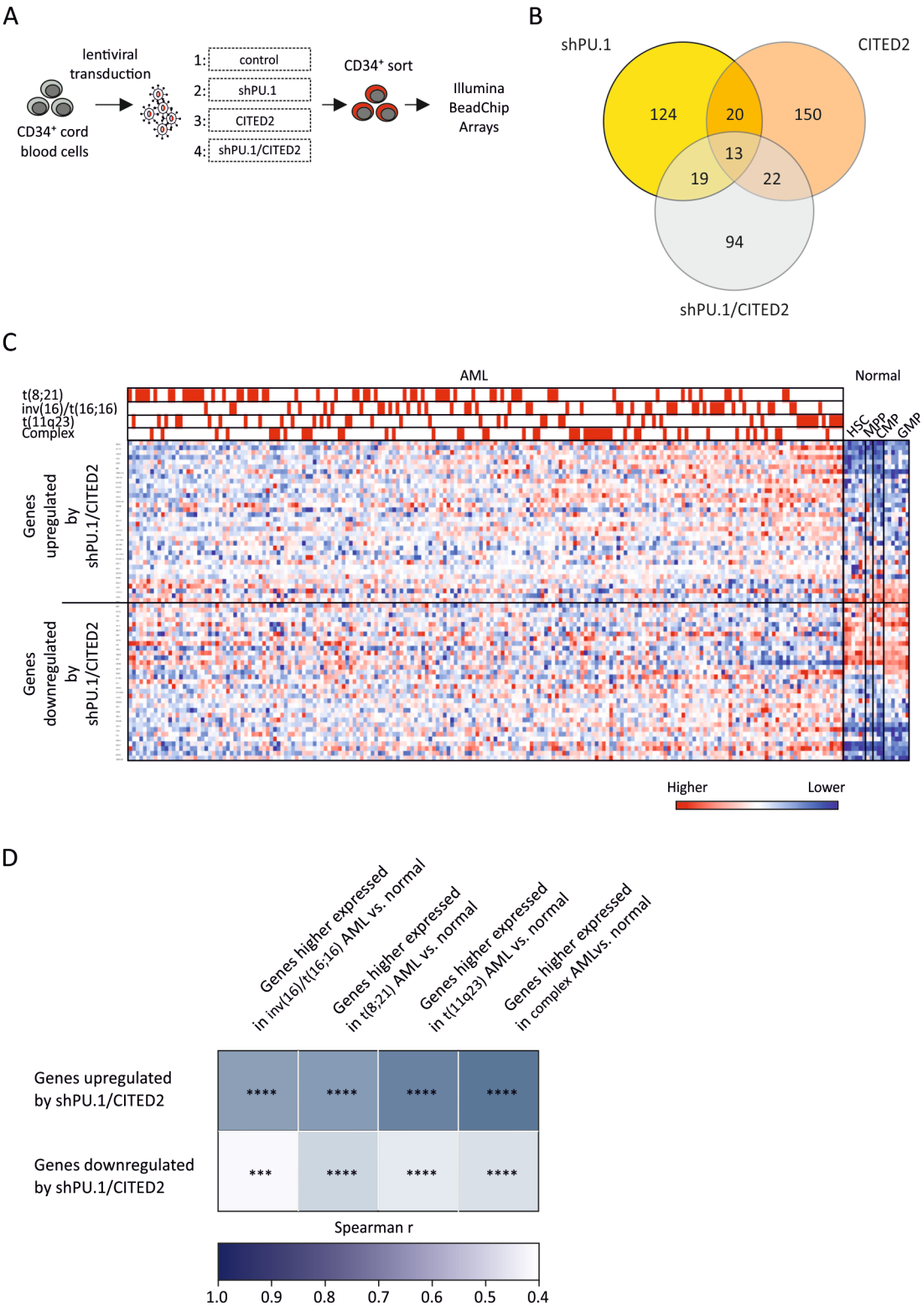
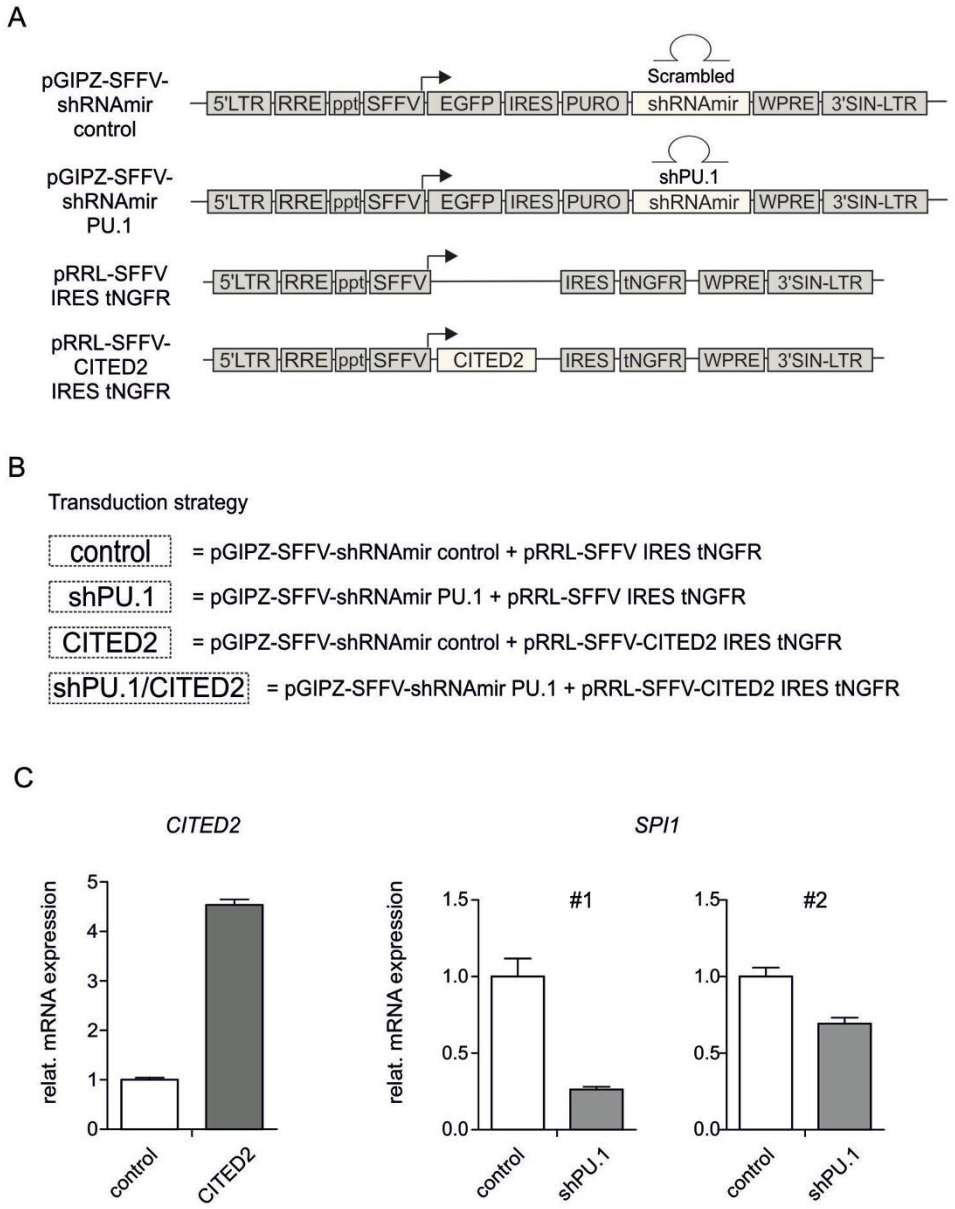

A
HSC donor
PU.1KD/KD
CD45.2
Irradiated NSG
recipient
CD45.1
CITED2-GFP or
control-GFP
lentivirus
B
Peripheral blood
Cohort A
% GFP in CD45.2 cells
injection
4
6
13
15
21
weeks after injection
*
Peripheral blood
Cohort B
control
CITED2
injection
4
11
16
20
weeks after injection
C
bone marrow
% GFP in CD45.2 cells
ns
control
CITED2
D
spleen weight (mg)
ns
control
CITED2

Figure 5


A
lentiviral transduction
CD34⁺ cord blood cells
1: control
2: shPU.1
3: CITED2
4: shPU.1/CITED2
CD34⁺ sort
Illumina BeadChip Arrays
B
shPU.1
CITED2
shPU.1/CITED2
124
20
150
13
19
22
94
C
AML
Normal
t(8;21)
inv(16)/t(16;16)
t(11q23)
Complex
HSC
MPP
CMP
GMP
Genes upregulated by shPU.1/CITED2
Genes downregulated by shPU.1/CITED2
Higher
Lower
D
Genes higher expressed in inv(16)/t(16;16) AML vs. normal
Genes higher expressed in t(8;21) AML vs. normal
Genes higher expressed in t(11q23) AML vs. normal
Genes higher expressed in complex AMLvs. normal
Genes upregulated by shPU.1/CITED2
Genes downregulated by shPU.1/CITED2
****
***
Spearman r
1.0
0.9
0.8
0.7
0.6
0.5
0.4

Mattes et al.

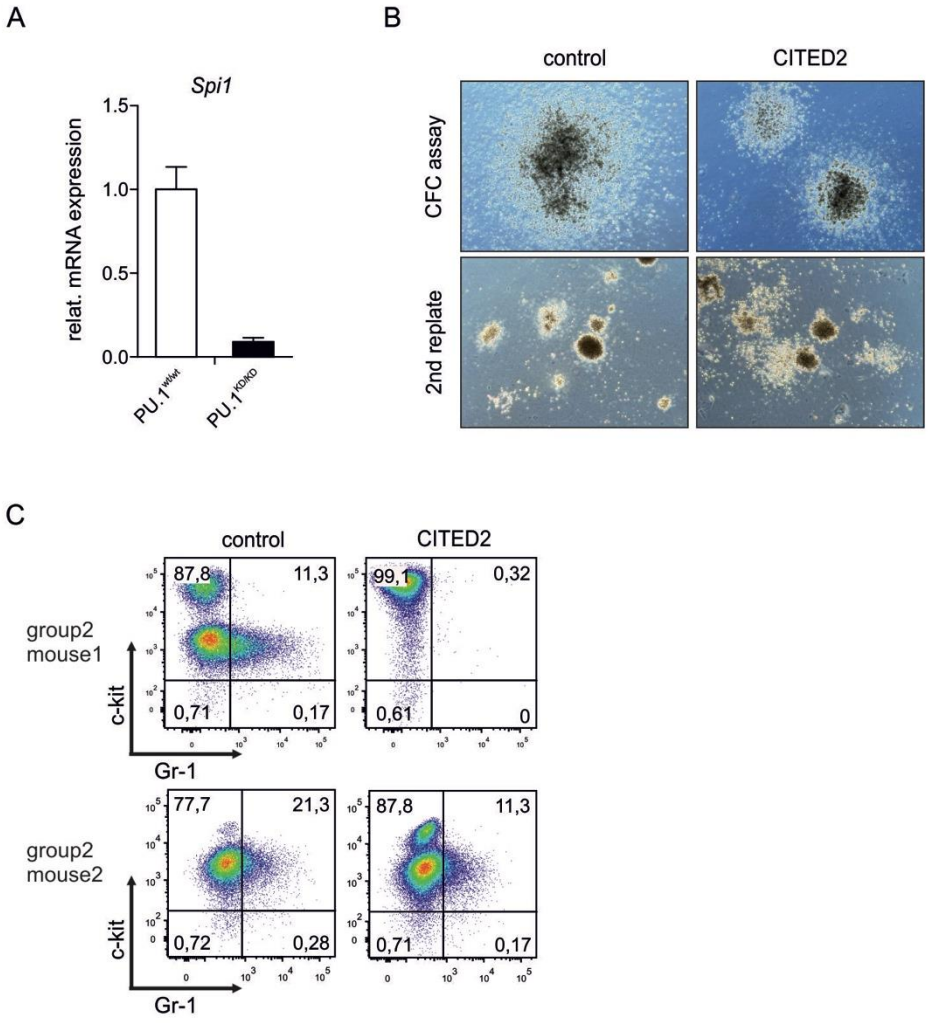
Supplementary data



Supplementary Figure S1 (Supplement to Figure 1)

Combined PU.1 down-regulation and CITED2-upregulation in CD34^{+} cord blood cells was mediated by lentiviral constructs

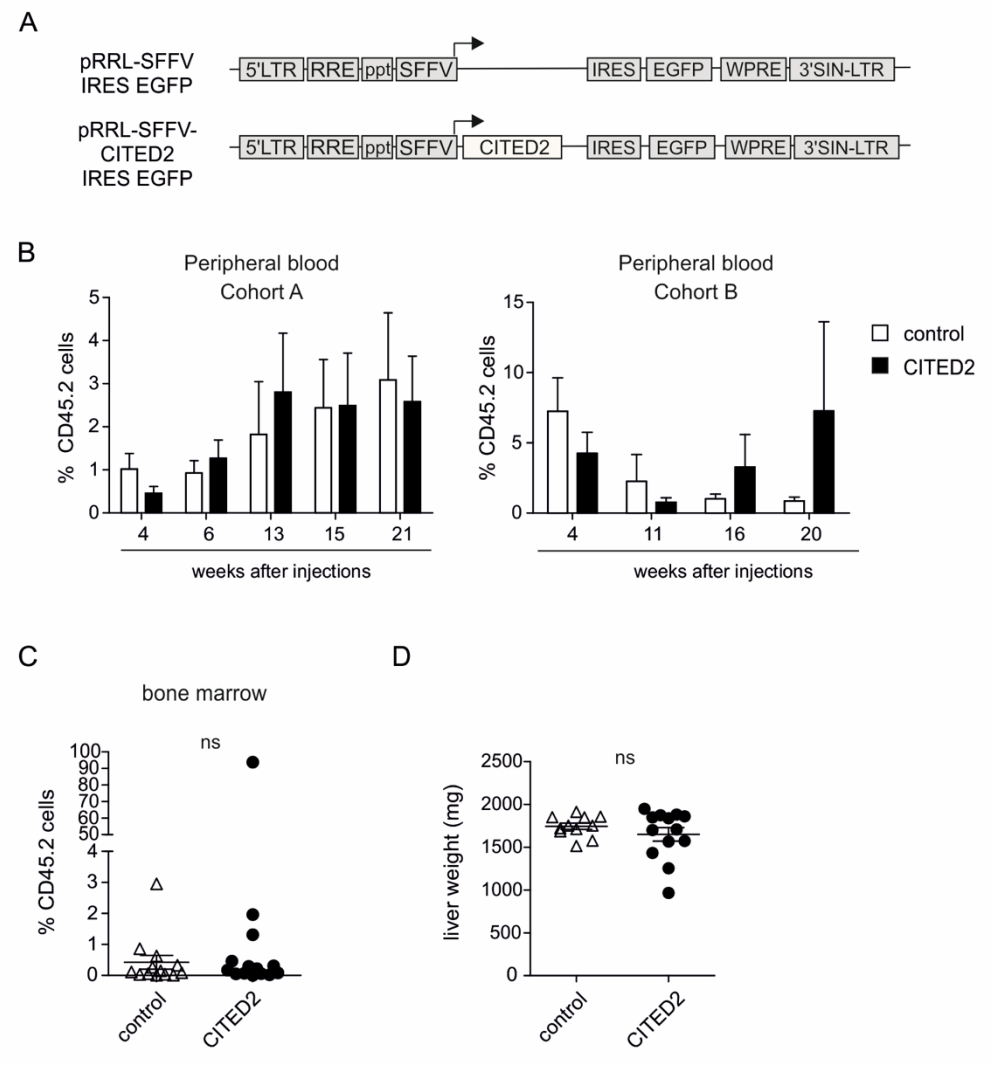
(A) Schematic overview of the lentiviral constructs used for transduction **(B)** Double transduction strategy with indicated constructs was performed to achieve either downregulation of PU1 (shPU.1), upregulation of CITED2 (CITED2) or a combination of both (shPU.1/CTED2). **(C)** Efficiency of lentiviral constructs was tested by Q-PCR using CD34^{+} cord blood cells that had been transduced with indicated constructs and sorted for double transduced cells. The level of *SPI1* (PU.1) downregulation varied between cord blood samples (#1, #2). Error bars represent s.d. of Q-PCR triplicates.



Supplementary Figure S2 (Supplement to Figure 3)

Overexpression of CITED2 in PU.1$^{KD/KD}$ cells

(A) Q-PCR for *Spi1* confirming an 80% reduction of *Spi1* (PU.1) expression in PU.1$^{KD/KD}$ BM cells compared to PU.1$^{WT/WT}$ cells. Error bars indicate s.d. of Q-PCR triplicates **(B)** Pictures show colonies from control- or CITED2 transduced PU.1$^{KD/KD}$ cells in primary CFC assays (upper panel) or 2nd round of replating. **(C)** FACS plots indicating c-Kit and Gr-1 expression of control- or CITED2 transduced PU.1$^{KD/KD}$ cells that have been harvested from CFC assay replates. Two samples of group 2, which is the group characterized by very high colony numbers in CFC assay replates, are shown.

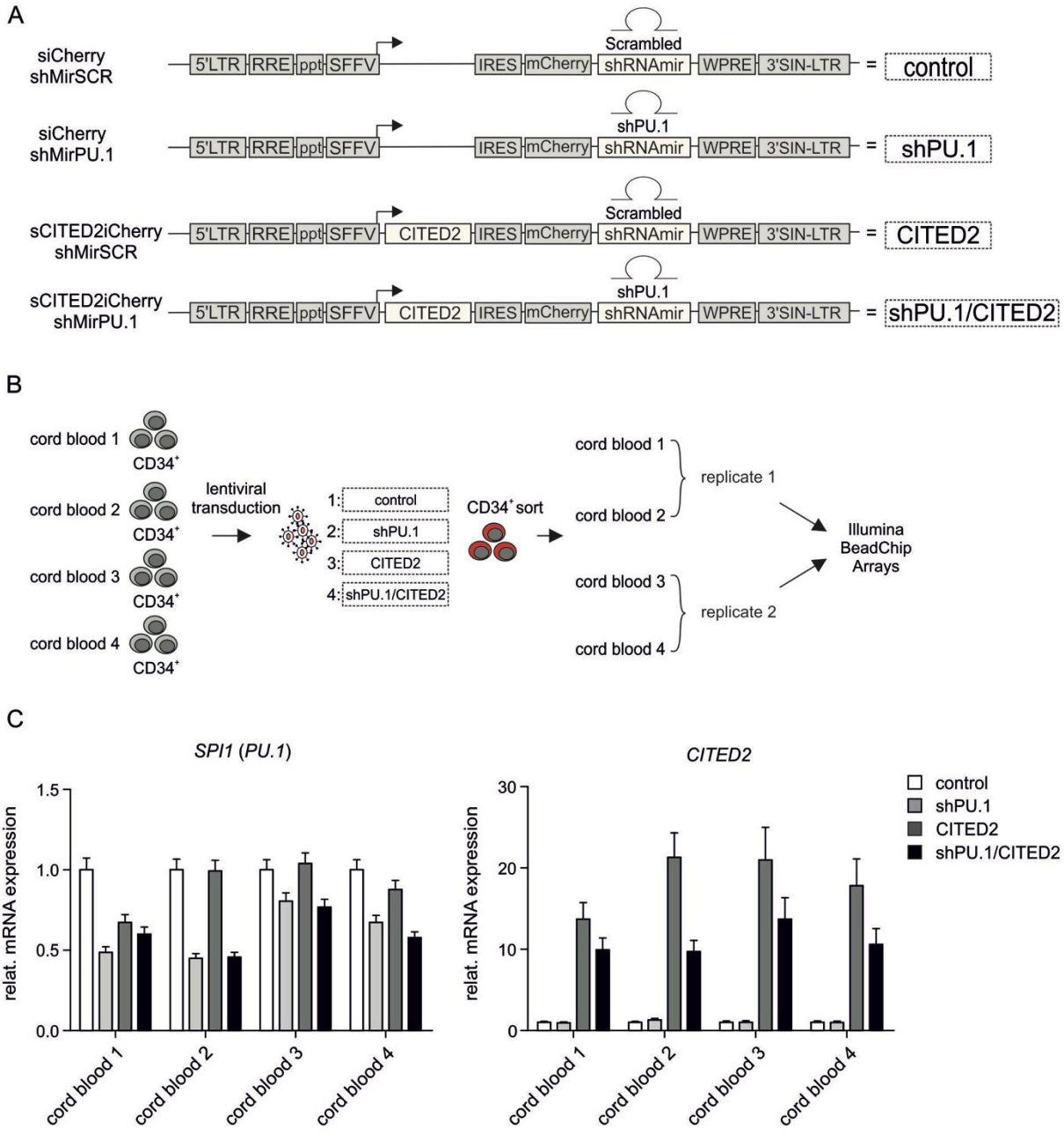


Supplementary Figure S3 (Supplement to Figure 4)

Overexpression of CITED2 in PU.1$^{KD/KD}$ HSCs is not sufficient for leukemic transformation

(A) Schematic overview of the lentiviral constructs used for transduction **(B)** Percentage of CD45.2 cells in peripheral blood after indicated number of weeks is shown. Error bars indicate s.d.; n=7 for each

cohort; **(C)** Percentage of CD45.2 cells in bone marrow of recipient mice at day of sacrifice 30-34 weeks after injection is shown. Error bars indicate s.d.; n=14; **(D)** Liver weights of sacrificed mice is indicated; Error bars indicate s.d.; n.s: not significant



Supplementary Figure S4 (Supplement to Figure 5)

Ilumina BeadChip array was performed with control-, shPU.1-, CITED2, and shPU.1/CITED2 CD34$^+$ CB cells

(A) Schematic overview of the lentiviral constructs used for transduction **(B)** Overview of the experimental procedure: 4 different cord blood samples were transduced with the indicated constructs to achieve either downregulation of PU1 (shPU.1), upregulation of CITED2 (CITED2) or a combination of both (shPU.1/CTED2). Samples with similar levels of *SPI1* downregulation were pooled and an lumina BeadChip array was performed with 2 replicates. **(C)** Levels of *SPI1* (PU.1) downregulation and CITED2 upregulation of transduced CB cells applied to the Ilumina BeadChip array were analyzed by Q-PCR. Error bars indicate s.d. of Q-PCR triplicates.

Supplementary Table S1

Table lists probesets that were found differentially expressed in shPU.1/CITED2- transduced CD34$^+$ cord blood cells compared to control transduced cells in an Ilumina BeadChip array.

Unique to shPU.1/CITED2		Common with CITED2		Common with shPU.1		Common in all groups	
ProbeID	**Gene Symbol**	**ProbeID**	**Symbol**	**ProbeID**	**Symbol**	**ProbeID**	**Symbol**
3610626	ADM2	2850041	B3Gn-T6	7330102	CSMD1	3710722	CCDC50
4390523	AGAP7	4060632	C17orf87	1660689	GRB14	2690328	LOC647357
2510368	ARID4A	3940435	EMP1	6330767	HDAC2	1400653	LOC651075
7650209	BMF	5810577	FGA	430403	HOXB6	1240139	LOC651680
3940152	C22orf40	1260341	IL13RA1	4880192	LOC100132771	110411	LOC653701
3870441	CAPN3	360672	LOC100130928	2600095	LOC165186	7650392	MIR1267
6220382	CD68	5670176	LOC642003	580575	LOC255620	4290450	PIK3R1
4040022	CD86	6450358	LOC644641	4640136	LOC648863	5260433	PMCHL1
2470196	DNAJB5	4590192	LOC645165	2690010	LOC650620	4830632	SLC7A7
940639	ERP27	2260608	LOC649422	3520240	LOC651137	5720682	TMEM176A
60307	FCF1	5670450	PURG	3120731	LOC653643	3610020	
6040598	GSDMB	1440730	SNORA29	6620725	PPAP2C	1430494	
4890279	HIST2H4A	450682	SNORA49	1340541	RGPD5	3850369	
3870102	HNMT	4180195	SP6	1400121	SLCO2B1		
2060553	HYALP1	630722	TBX19	4880519	SPO11		
5890021	IGSF11	1710273		2760242	TIAM1		
2470743	KMO	1030243		1300026			
610037	LAMA1	1990093		6770646			
3180609	LOC100008588	3460519		6960753			
6290142	LOC100008589	7560142					

2510446	LOC100130276	1070056
2750382	LOC100133916	2650048
6040386	LOC100134539	
5960240	LOC100134634	
1400717	LOC121456	
240204	LOC151457	
1580082	LOC158572	
5860255	LOC284296	
7150082	LOC285296	
6760093	LOC440386	
2450326	LOC642047	
1820519	LOC642838	
2850246	LOC643713	
1010543	LOC643888	
2000369	LOC644191	
5290685	LOC645183	
3870731	LOC645722	
7560484	LOC647827	
4810717	LOC650111	
5910674	LOC650698	
6370192	LOC728288	
3140358	LOC728787	
5700736	LOC729081	
3520259	LOC730202	
50332	MDFIC	
4810114	MGC12982	
70669	MIR1224	
990328	MS4A7	
3990477	MTHFR	
2480452	MYF5	
70008	NCF2	
2650113	NR1I2	
20068	OTUD1	
2340301	PION	
2070592	PPAPDC1A	
2630519	RSHL3	
1410440	SHC2	
4180647	SHPRH	
840189	SMTN	
1300349	SOCS4	
2370368	SON	

4120475	SOX15
7570670	SPIN1
4590767	SPRR1B
2350424	SUV420H2
4180521	TMEM63B
7040348	TMLHE
4830273	TWF1
5570546	U2AF1L4
1010500	UGP2
780180	USP9X
610438	
6510739	
4830300	
3940609	
2000709	
670725	
7380593	
4490139	
5090102	
1260470	
6480025	
830112	
3120300	
1580070	
630615	
3360170	
7610427	
5290433	
4050296	
240259	
4670491	
1990392	
7560402	

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57 Supplementary Table S2

58 Table lists primer sequences that have been used for Q-PCR.

name	forward primer 5'-3'	reverse primer 5'-3'

hHPRT	AGTTCTGTGGCCATCTGCTTAG	CGCCCAAAGGGAACTGATAGTC
hRPL27	TCCGGACGCAAAGCTGTCATCG	TCTTGCCCATGGCAGCTGTCAC
hRPS11	AAGATGGCGGACATTCAGAC	AGCTTCTCCTTGCCAGTTTC
hCITED2	CTTTGCACGCCAGGAAGGTC	CGCCGTAGTGTATGTGCTCG
hSPI1 (PU.1)	GCGACCATTACTGGGACTTC	ATGGGTACTGGAGGCACATC
mSPI1 (PU.1)	GCCTCAGTCACCAGGTTTCC	CCTTGTCCACCCACCAGATG3
mHPRT	AGTCCCAGCGTCGTGATTAG	CCAGCAGGTCAGCAAAGAAC
mB2M	TGACCGGCCTGTATGCTATC	GATCCCAGTAGACGGTCTTG

Supplementary Material and Methods

Lentiviral transduction

Human CITED2 cDNA was obtained through Addgene (plasmid 21487) and cloned into the multiple cloning site of either pRRL-SFFV-IRES-tNGFR or pRRL-SFFV-IRES-EGFP [33]. Vectors containing shRNA against SPI1 (PU.1) were obtained from GE Healthcare Dharmacon (#V3SVHS06_8494848) and the hairpin containing region was cloned into the pGIPZ-SFFV-EGFP-shRNAmir backbone using the MluI and NotI restriction enzyme sites. Constructs for combined CITED2 overexpression and PU.1 downregulation where obtained by cloning the shRNAmir cassette from the pGPIZ-SFFV-EGFP-shRNAmir vector into the pRRL-SFFV-IRES-mCherry backbone. Sequences and plasmids are available upon request. Lentiviral particles were produced as described before.[43] After 24 hours, medium was changed to HPGM and after 12 hours, supernatant containing lentiviral particles was harvested, concentrated using CentriPrep Ultracel YM-50 Filter Units (Merck Millipore) and stored at −80°C. Cells were transduced with lentiviral particles in the presence of 4 μg/ml polybrene in 2 consecutive rounds of 12 hours. During transduction, human $CD34^+$ cells were kept in HPGM supplemented with hSCF (Novoprotein), hFLT3 ligand (Celldex) and Npllate (Amgen) (100 ng/ml each). Murine bone marrow cells were kept in StemSpan SFEM (Stemcell Technologies) supplemented with 100 ng/ml mSCF (PepProtech), 100ng/ml hFLT3 ligand

(Celldex), 100ng/ml Nplate (Amgen) and 20 ng/ml mIL-3 (PepProtech). Cells were sorted 3 days after transduction on a MoFLo XDP or Astrios (DakoCytomation, Carpinteria, CA, USA) and applied to subsequent assays.

RNA isolation and Q-PCR

Total RNA was isolated using the RNeasy Micro Kit (QIAGEN) following manufacturer's instructions and reverse transcriped using the iScript cDNA synthesis kit (Bio-Rad). Real-Time PCR was performed on a CFX Connect System (Bio-Rad) using the SsoAdvanced SYBR Green Supermix (Bio-Rad). Data were quantified using CFX Manager software (Bio-Rad) and normalized to values of the housekeeping gene RPS11, RPL27, HPRT or B2M. Primer sequences are listed in Supplementary Table S2.

FACS analysis

Cells were sorted on a MoFLo XDP or Astrios (DakoCytomation, Carpinteria, CA, USA). All FACS analyses were performed on an LSRII (Becton Dickinson) flowcytometer and data was analyzed using FlowJo software. Murine lineage negative cells were selected using the Alexa Fluor 700 anti-mouse lineage cocktail (Biolegend, Uithoorn, The Netherlands, #133313). Antibodies used for flow cytometry of murine cells were: Alexa Fluor 488 anti-mouse Ly-6G/Ly-6C (Gr-1) (Biolegend, #108419), PE anti-mouse CD117 (c-kit) (Biolegend, #105807), Brilliant Violet 421 anti-mouse Ly6A/E (Sca-1) (Biolegend, #108127), PE/Cy7 anti-mouse/human CD11b (Biolegend, #101215).